DNA base adducts in urine and white blood cells of cancer patients receiving combination chemotherapies which include *N*-methyl-*N*-nitrosourea

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Urinary 3-methyladenine (3-MeAde) excretion and lymphocyte DNA adduct formation was studied in 15 patients receiving methylnitrosourea (MNU) at several dose levels (250 mg, 300 mg and 600 mg total dose, 143–385 mg m^{-2}) as part of various combination chemotherapies for advanced tumours (malignant melanoma, lymphoblastic lymphosarcoma and Hodgkin's disease). Urinary 3-MeAde levels were significantly increased over background in patients at all dose levels ($p < 0.001$) and the increases were dose-dependent ($r = 0.77$, $p < 0.01$). There were large interindividual variations in the excretion of 3-MeAde at each dose of MNU. In a subset of patients, *N*7-methyl-2′-deoxyguanosine (7-MedG) and *O*^{6}-methyl-2′-deoxyguanosine (*O*^{6}-MedG) levels in DNA from blood leucocytes showed dose-dependent increases, however there were no simple relationships between urinary methylated DNA bases and leucocyte DNA adducts. Levels of adducts in leucocyte DNA (7-MedG, < 17–217 μmol mol^{-1} dG; *O*^{6}-MedG, < 1.6–35 μmol mol^{-1} dG) were comparable with those reported for other methylating chemotherapeutic drugs. Leucocyte DNA and urinary methyl adducts may be useful markers of individual responses to treatment with methylating drugs.

Keywords: *3-methyladenine in urine, 7-methylguanine and O^6-methylguanine in blood DNA, chemotherapy,* N-methyl-N-nitrosurea, *cancer patients.*

Introduction

Epidemiological studies have demonstrated that second cancers can result from chemotherapy and/or radiotherapy used to treat the initial cancer (Kaldor *et al.* 1987). In the case of Hodgkin's lymphoma, which has a relatively good prognosis (75% 5-year survival), the cumulative risk of acute non-lymphocytic leukaemia can reach 15% at 15 years post-treatment (Kaldor *et al.* 1990). In order to reduce this incidence of treatment-induced cancers, it is important to understand which agents, combination of agents, or treatment protocols are responsible for second cancers. For many alkylating drugs it has proved difficult to separate the cytotoxic and tumorigenic consequences of the formation of various DNA adducts, as in the case of the nitrogen mustards (Povirk and Shuker 1994). An understanding of the molecular events which are responsible for therapeutic efficacy as opposed to those which lead to carcinogenesis could lead to modifications in chemotherapy protocols which maintain or increase efficacy whilst reducing the risk of second cancer.

Thus, in order to elucidate the role of particular alkylating drugs in second cancers the following points need to be addressed: (1) the relationship between drug dose and DNA adduct formation, (2) a comparison of the validity of different adducts as markers of exposure, (3) the influence of interindividual variations in DNA repair on adduct persistence, and (4) the relationship between the initial formation and persistence of DNA adducts and the induction of mutations both in somatic cells, e.g. lymphocytes and eventually in the second tumour (for example, mutation spectra). For therapies where long-term survival is good the predictiveness of DNA adducts for tumour risk could be addressed prospectively and the process of carcinogenesis could be studied.

In the wider context of studies on human exposure to carcinogens, the use of methylating agents such as procarbazine and dacarbazine and, very exceptionally, *N*-methyl-*N*-nitrosourea (MNU) in cancer chemotherapy is a rare situation where humans are exposed to well-defined doses of known carcinogenic agents. It affords the possibility to examine the potential value of DNA adducts as markers of human exposure to alkylating carcinogens.

This paper describes results from a study which was designed to address points (1) and (2) outlined above. Despite the wide use of some methylating agents such as procarbazine and dacarbazine, there have been relatively few reports of studies on DNA alkylation. A number of groups have reported increased levels of DNA methylation in peripheral lymphocytes of patients treated with procarbazine and/or dacarbazine (Souliotis *et al.* 1990, 1991, Mustonen *et al.* 1991, Van Delft *et al.* 1992, Lee *et al.* 1994). Urinary 3-MeAde has also been used as a non-invasive maker of DNA methylation in human volunteers (Prevost *et al.* 1990, 1993, Shuker and Farmer 1992, Prevost and Shuker 1996). However, there has hitherto been no attempt to use the two approaches in the same group of highly exposed human subjects and it is this novel aspect which is reported in this paper. Urinary 3-methyladenine (3-MeAde) and lymphocyte DNA methyl adducts were measured in patients receiving MNU as part of clinically indicated combination chemotherapy.

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MATERIALS AND METHODS

Selection of patients and collection of samples

Patients were included in the study if they received MNU as part of their combination chemotherapy. Combination chemotherapies including MNU were

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only used in cases where the disease was already well advanced and for which other treatment schedules had been used and found to be ineffective. All patients gave written consent for the therapy according to the Declaration of the 18th World Medical Assembly (1964) and treatment was performed with permission of the Ethical Committee of the N. N. Petrov Research Institute of Oncology. Permission for the use of MNU in cancer patients in combination with other drugs was given by the Committee of Pharmacology of the former Soviet Union.

MNU was administered by i.v. injection at total doses of 250, 300 or 600 mg according to previously described protocols (Emanuel *et al.* 1974) and patients received other alkylating cytostatic drugs including procarbazine (Table 1). For the purposes of the study described in this paper, urine and blood samples were collected as near to the beginning of each treatment, as often as was practicable. The actual collection days are indicated in Table 1.

Twenty-four-hour urine samples were collected into clean glass containers prior to and after the administration of MNU. Thus, each patient served as his or her own control. The total volume of the 24-h samples was measured and a 50 ml aliquot was collected, lyophilized and sent to IARC for urinary 3-MeAde analysis.

Blood samples (10 ml) were collected 4.5 h after the administration of MNU, the white blood cells were isolated by centrifugation using a Ficoll/Urografin mixture (final density 1.19 g ml^{-1}), frozen and sent to IARC for DNA isolation.

Urinary 3-MeAde determination

3-MeAde was determined in 2 ml aliquots of reconstituted lyophilized urine as described in Prevost *et al.* (1990). Briefly, the pH of aliquots of 2 ml was adjusted to 7.4, and [^{3}H]-3-MeAde (1000 dpm, as internal standard) was added. The urine was eluted through an immunoaffinity column (immobilized IgG from an anti-3-MeAde rabbit serum). After a series of washing steps (PBS and water), 3-MeAde was eluted with 1 M acetic acid (2 ml). One ml of the eluate was counted for radioactivity to calculate recovery and the remainder of the sample was evaporated to dryness and reconstructed in PBS for analysis by competitive ELISA.

7-MedG and O^6-MedG in lymphocyte DNA

Cells were resuspended in a total volume of 5 ml of DNA extraction buffer (10 mM Tris, pH 8.0; 400 mM NaCl; 2 mM EDTA) and 350 µl 10% SDS and 2 mg proteinase K were added. The mixture was incubated overnight at 37 °C. Proteins were precipitated by addition of 1.8 ml 6 M NaCl and removed by centrifugation. DNA was precipitated from the supernatant by adding 15 ml of ethanol and leaving at –20 °C for 2 h. Quantities of DNA ranged from 20 to 386 µg as judged by UV absorbance at 260 nm and those with 50 µg or more DNA were analysed for O^6-MedG and 7-MedG. These DNA samples were further purified by treatment for 1 h with RNAse A (50 µg) and RNase T1 (3 U) after dissolving in 500 µl of 1 mM Tris; 0.05 mM EDTA, pH 7.5. DNA was reprecipitated with ethanol, dried and then washed in 70% ethanol adjusted to pH 12 with 3 N NaOH and in order to effect imidazole ring-opening (*iro*) of 7-MedG adducts.

Analysis of O^6-MedG was by enzyme hydrolysis, Aminex A7 chromatographic purification and radioimmunoassay as previously described (Umbenhauer *et al.* 1985). However, in order to analyse 7-MedG the fractions from Aminex A7 chromatography containing the ring-opened form of this adduct (13–23 min), and the co-chromatographing thymidine, were collected. The fractions were dried under vacuum (Speed Vac, Savant, Farmingdale, NY, USA) and then subjected to affinity chromatography on *m*-aminophenylboronic acid–agarose to remove ribonucleosides as follows: samples were dissolved in 1 ml 0.2 M ammonium acetate and applied to the affinity columns (2 ml gel volume) which had been pre-equilibrated in the same buffer. A further 2 ml of the same buffer was added to the columns and the eluate containing the deoxynucleotides (7-MedG and dT) was collected, neutralized with 1 M acetic acid and dried under vacuum.

A reverse phase C18 column (24 cm × 0.46 cm) (Supelco, Bellefonte, PA, USA) with a C18 ODS guard column was used with a flow of 1 ml min^{-1} and the following gradient: (buffer A: H_2O; buffer B: methanol) 10 min 95% buffer A–5% buffer B; 10 min linear gradient to 80% buffer A–20% buffer B; 5 min linear gradient to 100% buffer B, 8 min wash 100% buffer B. *Iro* 7-MedG was eluted after 7–8 min and dT after 20 min. Fractions corresponding to *iro* 7-MedG were collected, dried under vacuum and analysed by ELISA, as previously reported (Degan *et al.* 1988). It was possible to detect 0.025–0.75 pmol *iro* 7-MedG per ELISA well, with 50% inhibition at about 0.2 pmoles. dT was quantitated by UV absorbance; the adduct concentration was calculated as pmol *iro* 7-MedG per µmol dT because the latter deoxynucleoside is present in all steps of the procedure and, therefore, is the most appropriate against which to express the level of 7-MedG. However, for clarity of presentation the data were converted to pmol *iro* 7-MedG per µmol dG using a dG : dT molar ratio of 0.65.

This method of measuring 7-MedG is applicable for high levels of DNA adducts in, for example, experimental studies using methylating agents, or in the current situation of therapies involving high doses of chemotherapeutic agents. More sensitive and specific methods have been developed for measurement of 7-MedG resulting from exposure to low levels of environmental methylating agents (Bianchini *et al.* 1993).

Statistical treatment of the results

The assumption used in the statistical analysis of the results was that the 3-MeAde content of the 24-h urines is an independent variable. This is true if the formation and metabolism of 3-MeAde is rapid. Preliminary studies established that excretion of 3-MeAde in human urine was essentially quantitative within 24 h following administration of deuterium-labelled analogues (Prevost *et al.* 1993) and repair of 3-MeAde in DNA is known to be extremely rapid in many experimental animals (Shuker and Farmer 1992). Thus, the experimental design used in this study corresponds to a standard 'within-subjects' or 'repeated-measures' design (Maxwell and Delaney 1990), which is amenable to straightforward analysis of the data. Statistical significance of the results was therefore determined by Student's *t*-test using Sigmaplot for Windows (v1.0) software (Jandel Scientific, Erkrath, Germany).

Results

Study population characteristics

Combination chemotherapies including MNU were used for three tumour types, namely, malignant melanoma, non-Hodgkin's and Hodgkin's lymphomas. The outcomes of treatment after each chemotherapeutic cycle are indicated according to the WHO classification (World Health Organization 1979, Table 1), although it should be noted that none of the patients survived for more than 6 months after the last treatment cycle.

Urinary 3-MeAde

In all 15 patients in this study, urinary 3-MeAde increased following treatment with MNU (250, 300 or 600 mg, Figure 1). Multiple samples were obtained from some patients (patients 67, 68, 70, 72, 73 and 74 [Table 1]) corresponding to different days in the treatment cycles. Samples 61 and 71 are from the same patient who received treatment cycles several months apart. The increases were highly significant: at 300 mg of MNU the mean 3-MeAde excretion was 143 nmol per day (SD 59.1, n = 14) post-treatment compared with 26.8 nmol per day (SD 19.5, n = 14) prior to treatment ($p < 0.001$). A similar result was seen at the 600 mg dose—post-treatment, 181 nmol 3-MeAde

Sample no.	Diagnosis	Schedule of Therapy	Sex	Age (years)	MNU dose (mg)	MNU dose (mg kg^{-1})	Day of treatment cycle	3-MeAde (nmol per 24h)	Response to treatment [c]
61-1	Malignant melanoma	MNU (600 mg i.v., 1[b]), Vcr (2 mg i.v., 1[b]),	F	44	0	0	−1	45.4	−
61-2		Bleo (10 mg i.v., 1–6)			600	8.57	1	169.0	
62-1	Malignant melanoma	MNU (600 mg i.v., 1), Vcr (2 mg i.v., 1),	F	38	0	0	−1	9.0	−
62-2		Bleo (10 mg i.v., 1–6)			600	9.23	1	186.1	
63-1	Malignant melanoma	MNU (600 mg i.v., 1), Vcr (2 mg i.v., 1),	F	62	0	0	−1	16.6	+
63-2		Bleo (10 mg i.v., 1–6)			600	8.33	1	30.3	
64-1	Lymphoblastic	MNU (300 mg i.v., 2), Cph (300 mg i.v., 1, 3, 5),	M	59	0	0	1	10.8	−
64-2	lymphosarcoma	Vcr (1.5 mg, 1), Predn (30 mg p.o., 1–7)			300	4	2	169.5	
65-1	Lymphoblastic	MNU (600 mg i.v., 2), Cph (300 mg i.v., 1, 3, 5),	M	49	0	0	1	11.4	−
65-2	lymphosarcoma	Vbl (8 mg, 1)			600	7.79	2	229.5	
66-1	Malignant melanoma	MNU (300 mg i.v., 1), Prosp (200 mg i.p., 2, 3, 4),	M	52	0	0	−1	8.7	−
66-2		Predn (15 mg, p.o., 1–4)			300	4.17	1	104.8	
67-1	Lymphoblastic	MNU (600 mg i.v., 2, 9), Cph (300 mg i.p., 1, 3, 5, 8,	M	63	0	0	1	38.7	−
67-2	lymphosarcoma	10, 12), Vbl (5 mg i.v., 1, 8), Predn (60 mg p.o.,			600	6.9	2	288.6	
67-3		1–12)			0	0	8	18.2	
67-4					600	6.9	9	156.3	
68-1	Hodgkin's disease	MNU (300 mg i.v., 1, 5, 8, 12), Vcr (1.5 mg i.v., 3, 10),	M	49	0	0	−1	12.4	+++
68-2		Pcz (150 mg p.o., 3, 4, 6, 9, 11, 13),			600	4.92	1	170.9	
68.3		Predn (30 mg p.o., 1–13)			0	0	11	44.7	
68-4					600	4.92	12	117.7	
69-1	Malignant melanoma	MNU (600 mg, 1), Vcr (2 mg i.v., 1),	F	48	0	0	−1	12.0	−
69-2		Bleo (15 mg i.v., 1–6)			600	11.11	1	247.9	
70-1	Hodgkin's disease	MNU (300 mg i.v., 1, 5, 8, 12), Vcr (1.5 mg i.v., 2, 9),	M	27	0	0	−1	15.3	+
70-2		Pcz (150 mg p.o., 2–15), Dexa (1.5 mg p.o., 1–15)			300	4.11	1	180.1	
70-3					0	0	11	74.6	
70-4					300	4.11	12	202.1	
71-1	Malignant melanoma	MNU (600 mg i.v., 1), Vcr (2 mg i.v., 1),	F	44	0	0	−1	13.2	+
71-2		Pep (10 mg i.v., 1, 3, 6)			600	8.57	1	142.3	
72-1	Hodgkin's disease	MNU (300 mg i.v., 1, 4, 9, 14), Vcr (1.5 mg i.v., 1, 9),	M	44	0	0	−1	37.7	+
72-2		Pcz (150 mg p.o., 1–14), Predn (40 mg p.o., 1–14)			300	3.26	1	122.6	
72-3					0	0	13	37.0	
72-4					300	3.26	14	44.3	
73-1	Hodgkin's disease	MNU (250 mg i.v., 6, 9, 13, 16), Vbl (8 mg i.v.,	F	13	0	0	5	25.3	−
73-2		1, 8, 15), Pcz (100 mg p.o., 7, 8, 10, 14, 17),			250	5.95	6	172.4	
73-3		Predn (40 mg p.o., 1–17)			0	0	15	11.4	
73-4					250	5.95	16	91.3	
74-1	Hodgkin's disease	MNU (300 mg i.v., 1, 5, 8, 12), Vbl (8 mg i.v., 7),	M	60	0	0	−1	37.3	+++
74-2		(Pcz 150 mg p.o., 2, 3, 6, 9, 10, 13),			300	4.76	1	181.2	
74-3		Predn (20 mg p.o., 1–13)			0	0	11	34.1	
74-4					300	4.76	12	107.7	
74-5		MNU (300 mg i.v., 1, 4, 8, 11), Vbl (8 mg i.v., 3),			0	0	−1	15.4	
74-6		Pcz (150 mg p.o., 2, 5, 6, 9, 10, 12, 13),			300	4.76	1	105.7	
74-7		Predn (20 mg p.o., 1–13)			0	0	10	34.4	
74-8					300	4.76	11	215.6	
75-1	Hodgkin's disease	MNU (300 mg i.v., 1, 5, 8, 12), Vbl (8 mg i.v., 7, 14),	M	28	0	0	−1	9.1	−
75-2		Pcz (150 mg p.o., 2, 3, 6, 9, 10, 13),			300	4.69	1	47.7	
76-1	Hodgkin's disease	Predn (15 mg p.o., 6–13),	M	50	0	0	−1	4.2	−
76-2		MNU (300 mg i.v., 1, 5, 8, 15), Vbl (8 mg, i.v., 3),			300	3.41	1	234.2	
		Pcz (150 mg p.o., 2, 4, 6, 9, 11, 13, 16),							
		Predn (30 mg p.o., 1–16)							

Table 1. *Individual details and results for MNU-treated patients.*

[a] MNU, *N*-methyl-*N*-nitrosourea; Bleo, bleomycin; Predn, prednisolone; Vcr, vincristine, Pep, peplomycin; Prosp, prospidin; Cph, cyclophosphamide; Pcz, procarbazine; Vbl, vinblastine.

[b] Day of treatment in cycle.

[c] − advancement; + stabilization; + + + partial remission..

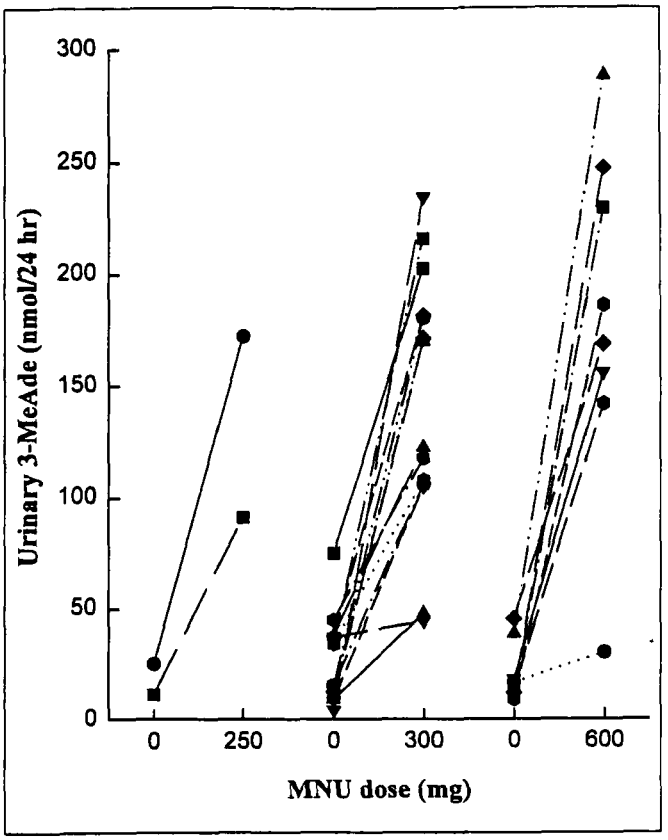

Figure 1. Urinary 3-MeAde excretion in cancer patients receiving MNU (250 mg, 300 mg or 600 mg). At each dose level the zero value was obtained for the 24-h period immediately preceding MNU administration.

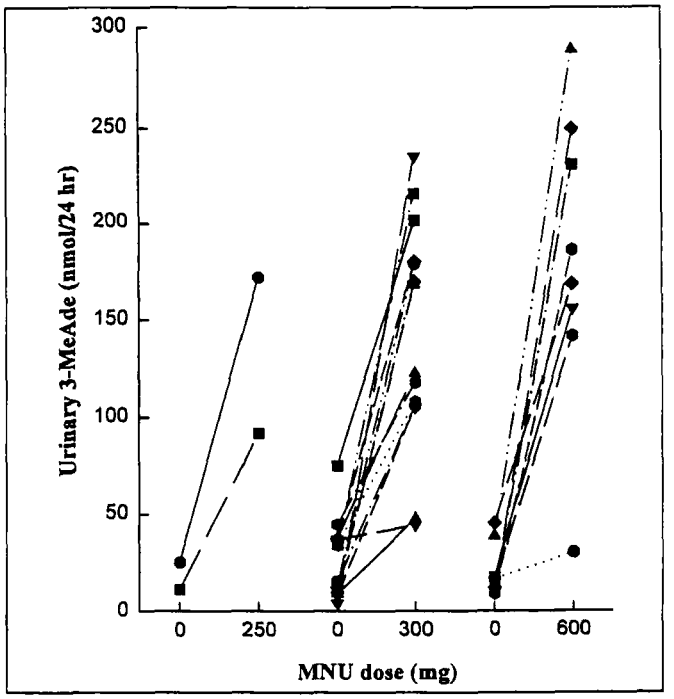

Figure 2. Dose–response for 3-MeAde excretion as a function of administered dose of MNU (mg m^{-2}). The values used for this correlation were based on urine samples which were collected at the beginning of treatment cycles when no other methylating agent had been administered. The values for 3-MeAde excretion at a zero dose of MNU are background values for each patient in urine samples collected immediately prior to treatment.

per day (SD 78.7, n = 10); pre-treatment, 20.6 nmol 3-MeAde per day (SD 13.7, n =8) ($p < 0.001$). As a few of the patients received procarbazine as well as MNU, some of the observed urinary 3-MeAde values reflect methylation from both drugs (see Discussion). In order to examine the dose–response relationship for MNU alone, 3-MeAde levels for the beginning of the treatment cycles only (except for patient 67, who did not receive procarbazine) were compared with dose of drug expressed as mg m^{-2} and a good correlation was obtained (r = 0.77, Figure 2).

Leucocyte DNA adducts

In eight patients (two of whom provided blood two samples from different treatment days), sufficient DNA was obtained from the buffy coat of the blood samples collected 4.5 h after MNU treatment to undertake analyses for 7-MedG and/or O^6-MedG. In some cases, the level of methyl DNA adducts was below the detection limit of the assays. Subsequent improvements in the assays have greatly increased sensitivity (Bianchini *et al.* 1993). However, insufficient DNA was available from the patients under study to allow reanalysis of the samples. In patients where adduct levels were quantifiable, the levels of both adducts increased with the dose of MNU (Figure 3). The ratio of O^6:$N7$ methyl adducts ranged from 0.12 to 0.45 in patients for which both adducts could be quantified in the same sample (Table 2). The higher ratios (0.43 and 0.45) were observed in patients who had also received repeated doses of procarbazine which is known to result in the accumulation of O^6-MedG (Souliotis *et al.* 1990).

Discussion

In early studies on alkylating cystostatic drugs, a number of methylating *N*-nitroso compounds including MNU (NSC-23909) were tested and showed antitumour activity in *in vitro* and animal screening assays (reviewed by Montgomery [1981] and Mitchell and Schein [1986]). Many analogues of MNU were synthesized and several (such as bis-chloroethylnitrosourea [BCNU, NSC-409962]) passed into widespread use, particularly in the treatment of brain tumours (Mitchell and Schein 1986). However, MNU itself continued to attract interest due to its consistent activity against various experimental tumours and clinical trials began in the former Soviet Union in 1966 (Emanuel *et al.* 1974). Since that time MNU has been used in single agent palliative therapy in undifferentiated carcinoma of the lung and Hodgkin's disease (Emanuel *et al.* 1974) and as part of a number of combination chemotherapies in treatment of various advanced solid tumours (Kolaric 1977, Gershanovich *et al.* 1988, Tanayev and Gershanovich 1991). Other chemotherapeutic drugs which are

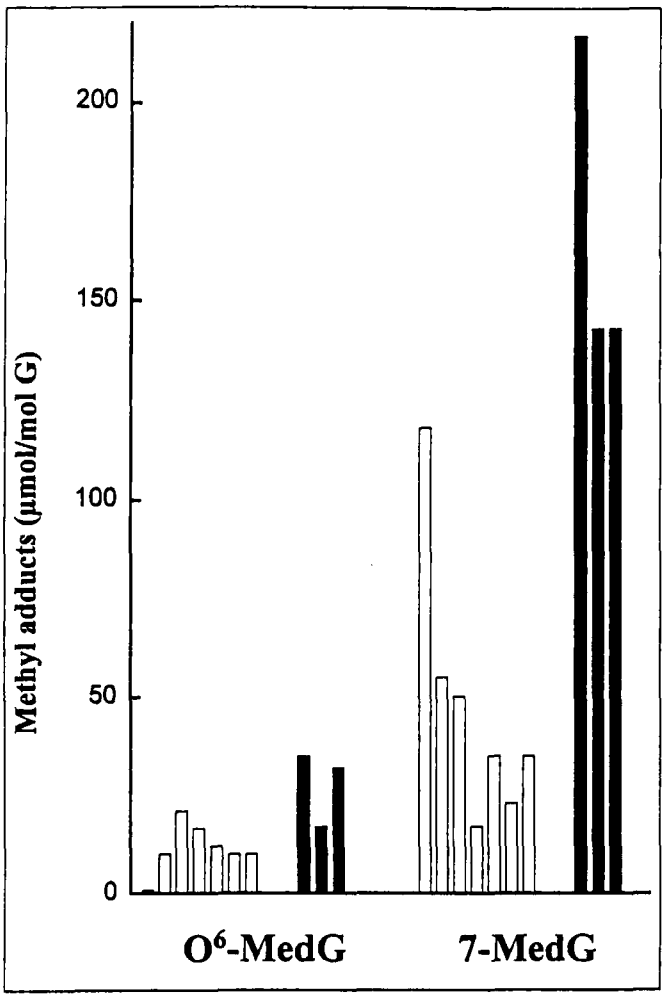

Figure 3. 7-MedG adduct levels in leucocyte DNA from blood collected from a subset of patients receiving MNU at 300 mg (open bars) or 600 mg (filled bars). The values were taken from Table 2.

methylating agents, and which have been wisely used in combination chemotherapies, include procarbazine and dacarbazine.

In the group of patients reported in this paper, MNU was always administered in combination with other cytostatic drugs, including procarbazine and cyclophosphamide, on other days of the treatment cycle (Table 1). However, urinary excretion of 3-MeAde clearly increased in all the patients following administration of MNU and was related to the dose (Figures 1 and 2). The magnitude of the increase varied considerably within the treatment groups with patients excreting from 13.7 to 250 nmol 3-MeAde per day over background. Previous studies have shown that humans excrete basal levels of 3-MeAde as a consequence of its presence in the diet (Prevost *et al.* 1990, 1993) which precludes the use of this adduct as a marker of low level exposure to methylating agents. However, in this study each patient served as his or her own control and the dose of MNU was relatively large (143–385 mg m^{-2}) which led to the observed response. Remarkably large individual variations in the level of urinary 3-MeAde were observed at the different dose levels (Figure 1), considering that MNU does not require metabolic activation to be converted into a methylating agent. As the kinetics of repair and excretion of 3-MeAde in humans is rapid ($t_{1/2}$ = *ca* 4 h [Prevost *et al.* 1993]), this variation in 24-h excretion levels probably reflects interindividual differences in the extent of DNA alkylation which could be influenced by a number of factors such as the concomitant administration of other chemotherapeutic drugs or the clinical status of the patient.

In those patients for whom sufficient DNA was available (n = 8), levels of *N*7-MedG and *O*⁶-MedG increased with increasing doses of MNU (Figure 3). Blood samples were not available from patients prior to treatment, but in previous studies of Hodgkin's disease and metastatic melanoma, patients' pre-treatment levels of *N*7-medG and *O*⁶-medG were generally below 1 and 0.1 µmole adduct per mole dG respectively (Souliotis *et al.* 1990, Philip *et al.* 1996).

An attempt was therefore made to examine the relationship between leucocyte DNA adducts and urinary 3-MeAde in those patients for whom DNA was available. There were only weak correlations between urinary 3-MeAde excretion and both 7-MedG (r = 0.27) and *O*⁶-MedG (r = 0.56) levels between individuals (data not shown). However, it is worth emphasizing that the different end points represent quite

Sample no.	Dose (mg MNU)	MNU (mg kg⁻¹)	Day (dose no.)	*O*⁶-MedG (µmol mol⁻¹ G)	7-MedG (µmol mol⁻¹ G)	Ratio (*O*⁶/*N*7)
62-2	600	9.23	1(1)	35.1	217.0	0.16
63-2	600	8.33	1(1)	16.8	142.6	0.12
64-2	300	4.00	2(1)	<1.6	118.2	n.d.[a]
65-2	600	7.79	2(1)	31.9	142.6	0.22
70-2	300	4.11	1(1)	10.1	54.9	0.18
70-4	300	4.11	12(4)	21.2	49.7	0.43
72-4	300	3.26	14(4)	16.5	<16.7	n.d.
74-2	300	4.76	1(1)	12.0	<34.8	n.d.
74-4	300	4.76	12(5)	10.3	22.6	0.45
75-2	300	4.69	1(1)	10.0	<35.7	n.d.

Table 2. Leucocyte methyl-DNA adducts in 10 samples from eight patients treated with MNU.

[a] n.d., not done because one of the values was at the limit of detection.

different parameters: blood was collected at 4.5 h post-dosing, and the level of methyl adducts present at that time represents the result of initial DNA modification and repair (glycosylases for 7-MedG and 3-MedA, and alkyltransferase for O^6-MedG). The kinetics of repair for each of the adducts are quite different (Den Engelse *et al.* 1986). Interestingly, one patient had elevated O^6-alkylguanine transferase levels and undetectable levels of O^6-MedG in leucocytes (data not shown). In this study, 3-MeAde was measured in 24-h urine samples and represents the integration of formation, repair and excretion over that time period. Given the differences in time of sample collection and the kinetics of adduct formation and loss, it is not surprising that no simple relationship exists between urinary levels of 3-MeAde and the levels of *N*7-MedG or O^6-MedG in leucocytes.

A number of studies have been reported in which DNA adducts have been measured in circulating leucocytes in patients receiving methylating chemotherapeutic drugs and these are summarized in Table 3. MNU gives rise to similar levels of methyl adducts per mole of drug to those obtained from dacarbazine, which is also administered as a single dose (Van Delft *et al.* 1992, Lee *et al.* 1994, Philip *et al.* 1996).

In a study of mammary carcinogenesis in rats both MNU and procarbazine gave rise to 7-MedG and O^6-MedG in all tissue analysed although MNU gave five times more 7-MedG on a mol kg^{-1} basis than procarbazine (Fong *et al.* 1990, 1992). Consequently, the procarbazine used in combination with MNU in the current study is likely to have made a contribution to the measured DNA adduct levels for samples collected later in the treatment cycles (for example, samples 70-2 and 70-4 in Table 3). This effect was also reflected in urinary 3-MeAde excretion in the same patient (Table 1).

There have been a number of experimental studies aimed at establishing the relationship between DNA methylation in target organs and peripheral lymphocytes from a range of methylating agents which all give rise to the same reactive intermediate but which display quite different target organ selectivity. It is worthwhile emphasizing that, in contrast to many of the methylating agents which have been studied, MNU does not require metabolism to become a methylating agent but simply decomposes in aqueous solution to give the methyl diazonium ion (Montgomery 1981). The administration of the hepatocarcinogen dimethylnitrosamine (DMN) to rats gave rise to similar levels of 7-MedG in blood leucocytes and the liver (Degan *et al.* 1988). Fong *et al.* (1990) found that levels of 7-MedG in blood leucocytes of pigs were similar to adduct levels in the liver after multiple doses of procarbazine and the leucocyte levels were in turn very similar to those reported in humans (Mustonen *et al.* 1991). A striking correlation was seen between 7-MedG in DNA from blood leucocytes and liver, in individual animals, after treatment with different doses of several methylating carcinogens (Bianchini and Wild 1994a, b), however, the ratio between 7-MedG in target organs and blood leucocytes was highly variable depending on the carcinogen administered (Bianchini and Wild 1994b).

In contrast to studies in experimental animals, the measurement of DNA methylation in internal target organs in humans is obviously extremely difficult and some indirect measure of these parameters is desirable. In addition to the measurement of DNA adducts in blood, the quantitation of excreted DNA adducts in urine has been explored as one approach to this problem (Shuker and Farmer 1992). In this small study, we have shown that urinary 3-MeAde increased in every patient treated with MNU but that there was considerable interindividual variation.

Interestingly, methylating agents derived from MNU have been developed for clinical use (e.g. streptozotocin

Drug	Dose[a]	Subjects	7-MedG (μmol mol^{-1} G)	O^6-MedG (μmol mol^{-1} G)	Reference
Dacarbazine	300 mg (3.23–5.77 mg kg^{-1})	11	ND[e]	Up to 0.72[b]	Souliotis *et al.* (1991)
Dacarbazine	380–1600 mg (225–800 mg m^{-2})	7	40–120[c]	ND	van Delft *et al.* (1992)
Dacarbazine	400 mg m^{-2}	8	ND	0.7–14.3[f]	Lee *et al.* (1994)
Procarbazine	1050–2800 mg	3	15.5–48.5	ND	Mustonen *et al.* (1991)
Dacarbazine	1200 mg	1	14.0		
Procarbazine	1500 mg (total accumulated dose)	7	ND	0.2–0.45[b]	Souliotis *et al.* (1990)
MNU	300 mg	8	< 17–118	< 1.6–21	This paper
	600 mg		143–217	17–35	

Table 3. Methylated adducts in leucocyte DNA of humans treated with methylating chemotherapeutic drugs.

[a] The dose is expressed in the units reported in the reference.

[b] Quantitated by competitive repair assay.

[c] Measured by ELISA, data originally presented as 7-MedG per 10^6 nucleotides.

[d] Measured by ^{32}P-postlabelling and originally expressed as adducts per 10^7 nucleotides.

[e] ND, not determined.

[f] Values for first cycle of treatment.

[Montgomery 1981]) and are still being evaluated as chemotherapeutic agents (e.g. aranoza [Perevodchikova *et al.* 1992]). The methodology described in this paper may prove useful as an indication of individual response to methylating drugs and is being actively explored in the case of procarbazine and dacarbazine which are used alone or in combination with other drugs in the treatment of metastatic melanoma (Cocconi *et al.* 1992). Recent results with patients receiving high-dose procarbazine for the treatment of metastatic melanoma indicate that urinary 3-MeAde is increased over background and shows a maximum of excretion between 4 and 20 h post-administration (Shuker, Crawley, Braybrooke and Harris, unpublished observations).

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